Dynamic Architecture of Eukaryotic DNA Replication Forks In Vivo, Visualized by Electron Microscopy

Zellweger, Ralph ; Lopes, Massimo

Abstract: The DNA replication process can be heavily perturbed by several different conditions of genotoxic stress, particularly relevant for cancer onset and therapy. The combination of psoralen crosslinking and electron microscopy has proven instrumental to reveal the fine architecture of in vivo DNA replication intermediates and to uncover their remodeling upon specific conditions of genotoxic stress. The replication structures are stabilized in vivo (by psoralen crosslinking) prior to extraction and enrichment procedures, allowing their visualization at the transmission electron microscope. This chapter outlines the procedures required to visualize and interpret in vivo replication intermediates of eukaryotic genomic DNA, and includes an improved method for enrichment of replication intermediates, compared to previously used BND-cellulose columns.

DOI: https://doi.org/10.1007/978-1-4939-7306-4_19

Chapter 19

Dynamic Architecture of Eukaryotic DNA Replication Forks In Vivo, Visualized by Electron Microscopy

Ralph Zellweger and Massimo Lopes

Abstract

The DNA replication process can be heavily perturbed by several different conditions of genotoxic stress, particularly relevant for cancer onset and therapy. The combination of psoralen crosslinking and electron microscopy has proven instrumental to reveal the fine architecture of in vivo DNA replication intermediates and to uncover their remodeling upon specific conditions of genotoxic stress. The replication structures are stabilized in vivo (by psoralen crosslinking) prior to extraction and enrichment procedures, allowing their visualization at the transmission electron microscope. This chapter outlines the procedures required to visualize and interpret in vivo replication intermediates of eukaryotic genomic DNA, and includes an improved method for enrichment of replication intermediates, compared to previously used BND-cellulose columns.

Key words Electron microscopy, DNA replication, Psoralen crosslinking, In vivo replication intermediates, Replication fork reversal, ssDNA, Nucleosome position

1 Introduction

Visualizing DNA replication intermediates (RIs) by structural approaches has proven invaluable to complement standard cell and molecular biology studies on the DNA replication process. Owing to the high magnification that can be achieved, transmission electron microscopy (EM) has helped uncovering the fine architecture of DNA replication forks. Since its original establishment by Dr. José Sogo at the ETH Zurich, this EM approach improved our understanding of the physiological process of genomic or episomal DNA duplication, in different model systems ranging from bacteriophages to mammalian cells [1–4]. The same approach was later extensively used to investigate specific conditions of replication stress in yeast cells, revealing profound changes of RI architecture upon fork stalling and other types of genotoxic stress [5–9]. More recently, several studies have made use of this technique in higher eukaryotic systems, uncovering the remodeling of replication forks

Marco Muzi-Falconi and Grant W. Brown (eds.), *Genome Instability: Methods and Protocols*, Methods in Molecular Biology, vol. 1672, DOI 10.1007/978-1-4939-7306-4_19,

as a global response to cancer-relevant replication stress and identifying cellular factors orchestrating such a response [10–19]. Overall, this approach has played a pivotal role in providing structural insight in the DNA replication process and has showed the potential to confirm, disprove, or refine long-standing models and dogmas in this field.

This chapter will focus on the experimental procedures required to extract genomic RIs from yeast, mammalian cells, and *Xenopus* egg extracts and analyze their fine architecture in vivo using the Transmission Electron Microscope (TEM). We refined several experimental steps of previously published protocols [20, 21]. In particular, we optimized a new protocol for RI enrichment (*see* Subheading 3.7), which no longer relies on BND cellulose and which showed improved enrichment of RIs over linear non-replicating DNA fragments, increasing the speed of the analysis. The technique described here is in principle reproducible in any lab, provided that a TEM and a proper high vacuum evaporator of carbon and platinum/carbon are available.

A crucial prerequisite for these investigations is in vivo psoralen crosslinking of DNA, achieved by repetitive exposure of living cells to tri-methyl-psoralen (TMP), followed by irradiation pulses with 365–366 nm monochromatic light. In vivo psoralen crosslinking is critical for two reasons (Fig. 1): (a) the formation of inter-strand crosslinks induced by this treatment prevents branch migration of cellular DNA and generally stabilizes in vivo RIs during DNA extraction and de-proteinization; (b) the inaccessibility of nucleosomal DNA to the crosslinks provides an opportunity (by proper modification of the technique, *see* Subheading 3.9.2) to obtain important information on nucleosome positioning in vivo and nucleosome dynamics at DNA replication forks [2, 5, 22–24]. Under denaturing conditions, DNA strands are separated wherever a nucleosome was positioned in vivo and are kept together by the interstrand crosslinks induced in the linker DNA (Fig. 1), giving to double-stranded DNA (dsDNA) the appearance of a string of single-stranded DNA (ssDNA) bubbles (Figs. 1 and 8). In fact, this technique has not only been coupled to EM, but also to more standard molecular biology approaches to study in vivo chromatin structure [25].

After in vivo-psoralen crosslinking, genomic DNA is extracted from the cells by standard procedures, minimizing mechanical forces to avoid the shearing of chromosomal DNA. DNA replication intermediates are then enriched by binding, washing, and elution in a QIAGEN-genomic tip 20 column, taking advantage of the high affinity of this resin to ssDNA, which is invariably present at DNA replication forks. With optimal stringency, this enrichment procedure allows an approximately 20-fold enrichment of RIs, although the majority of the recovered molecules are still linear duplex DNA (*see* Subheading 3.11, **step 3**). The non-

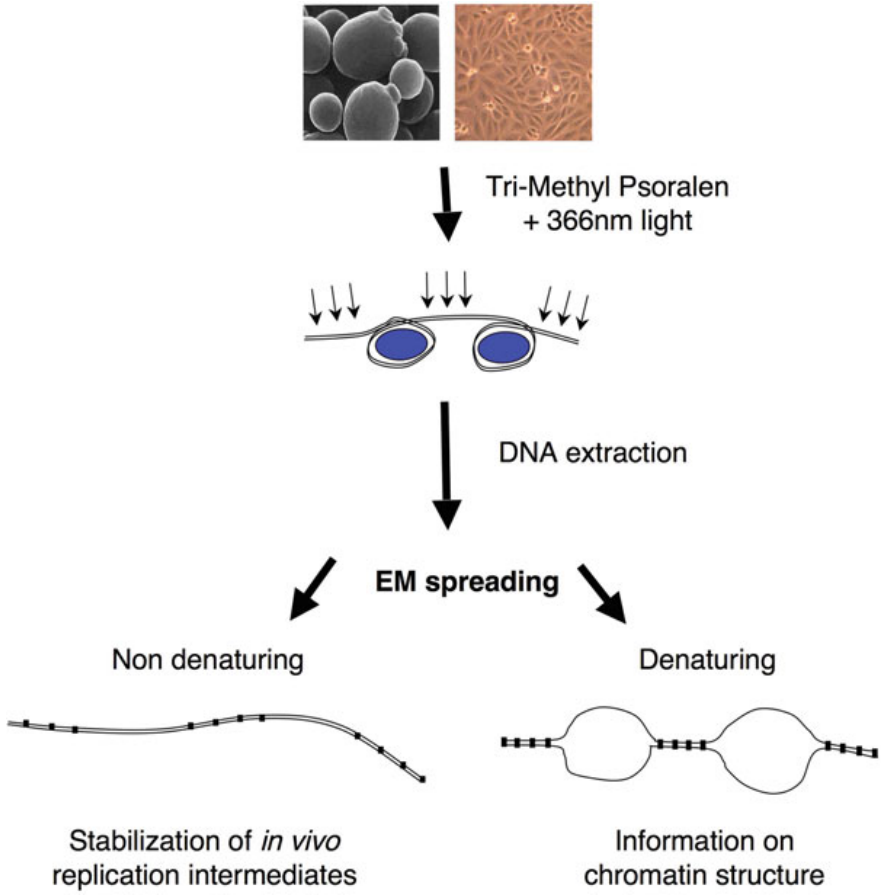

Fig. 1 Graphic representation of the psoralen crosslinking procedure and advantages for studies on DNA replication intermediates and nucleosome dynamics (*see* Subheading 1 for details). For the sake of clarity, linker- and nucleosomal-DNA are not drawn to scale in the graphical representation

saturating conditions described here for RI enrichment exclude that the recovered RI pool is biased toward molecules containing longer ssDNA stretches.

Once the DNA sample is enriched for RIs, it is usually concentrated in size-exclusion columns and used for protein-free DNA spreadings in the presence of the cationic detergent benzyldimethylalkylammoniumchloride (BAC), using water as hypophase, with a modified version of the method originally described by Vollenweider et al. [26]. The low molecular weight of this spreading agent (compared to protein-based methods) generally allows a better visualization of details (i.e., secondary structures) along the DNA molecules and an easier identification of ssDNA regions.

The monolayer nucleic acid film is then absorbed to freshly prepared carbon-coated grids and stained with uranyl acetate to improve the contrast of DNA molecules (in particular of ssDNA, [26, 27]). The grids are then subjected to flat angle-rotary shadowing with Platinum, which allows for the visualization of individual DNA molecules over the background granularity of the carbon

support. The reproducibility of the shadowing angle is a crucial point for the success of this technique. Once a high quality sample is obtained (in terms of molecule concentration, unfolding, and contrast over the background), a high number of RIs are photographed and carefully analyzed for important structural features (presence of ssDNA, secondary structures, nucleosome dynamics, etc.). Contour length measurements on the digital files complement the visual investigation and lead to accurate statistical analysis of the RI population analyzed.

2 Materials

2.1 In Vivo Psoralen Crosslinking (S. cerevisiae)

1. Ice-cold deionized water (*S. cerevisiae*).
2. Standard Petri dish, diameter 8.5 cm (*S. cerevisiae*).
3. Ice-cold 1× PBS (mammalian cells).
4. 96-Well plates (Xenopus egg extracts).
5. Ice-cold EB buffer (Xenopus egg extracts).
6. Tissue culture dishes, 60 × 15 mm (mammalian cells).
7. 4,5′,8-Trimethylpsoralen (TMP) stock solution: dissolve 200 μg/ml TMP (Sigma, cat. T6137) in Ethanol 100%. Stir extensively until the compound is dissolved completely. The solution can be stored at 4 °C for at least 1 year. Stir briefly at RT before each usage. Due to its DNA-modifying potential, TMP-containing solutions should be handled with gloves, labcoat, and protection glasses.
8. Biolink Crosslinker 365 nm, cat. B89110 (*see* **Note 1**).
9. Monochromatic 365 nm lamps for Biolinker (replacement UV bulbs, 8 W, cat. B89270).
10. UVP UVX Radiometer, with 365 nm sensor.
11. Freezing pack, flat, 1.5–2 cm thick.
12. Flat metal support (1 cm), precooled at −20 °C. The surface should be large enough to accommodate several Petri dishes (to crosslink more samples at the same time), but small enough to be easily inserted and removed from the available Crosslinker. This support is placed on the top of the freezing pack, to prevent heating of the samples during the incubation/irradiation cycles required for psoralen crosslinking.

2.2 In Vivo Psoralen Crosslinking (Mammalian Cells)

1. Ice-cold deionized water (*S. cerevisiae*).
2. Standard Petri dish, diameter 8.5 cm (*S. cerevisiae*).
3. Ice-cold 1× PBS (mammalian cells).
4. 96-Well plates (Xenopus egg extracts).

5. Ice-cold EB buffer (Xenopus egg extracts).
6. Tissue culture dishes, 60 × 15 mm (mammalian cells).
7. 4,5′,8-Trimethylpsoralen (TMP) stock solution: dissolve 200 μg/ml TMP (Sigma, cat. T6137) in Ethanol 100%. Stir extensively until the compound is dissolved completely. The solution can be stored at 4 °C for at least 1 year. Stir briefly at RT before each usage. Due to its DNA-modifying potential, TMP-containing solutions should be handled with gloves, lab-coat, and protection glasses.
8. Biolink Crosslinker 365 nm, cat. B89110 (*see* **Note 1**).
9. Monochromatic 365 nm lamps for Biolinker (replacement UV bulbs, 8 W, cat. B89270).
10. UVP UVX Radiometer, with 365 nm sensor.
11. Freezing pack, flat, 1.5–2 cm thick.
12. Flat metal support (1 cm), precooled at −20 °C. The surface should be large enough to accommodate several Petri dishes (to crosslink more samples at the same time), but small enough to be easily inserted and removed from the available Crosslinker. This support is placed on the top of the freezing pack, to prevent heating of the samples during the incubation/irradiation cycles required for psoralen crosslinking.

2.3 Psoralen Crosslinking of Sperm DNA (Xenopus Egg Extracts)

1. Ice-cold deionized water (*S. cerevisiae*).
2. Standard Petri dish, diameter 8.5 cm (*S. cerevisiae*).
3. Ice-cold 1× PBS (mammalian cells).
4. 96-Well plates (Xenopus egg extracts).
5. Ice-cold EB buffer (Xenopus egg extracts).
6. Tissue culture dishes, 60 × 15 mm (mammalian cells).
7. 4,5′,8-Trimethylpsoralen (TMP) stock solution: dissolve 200 μg/ml TMP (Sigma, cat. T6137) in Ethanol 100%. Stir extensively until the compound is dissolved completely. The solution can be stored at 4 °C for at least 1 year. Stir briefly at RT before each usage. Due to its DNA-modifying potential, TMP-containing solutions should be handled with gloves, lab-coat, and protection glasses.
8. Biolink Crosslinker 365 nm, cat. B89110 (*see* **Note 1**).
9. Monochromatic 365 nm lamps for Biolinker (replacement UV bulbs, 8 W, cat. B89270).
10. UVP UVX Radiometer, with 365 nm sensor.
11. Freezing pack, flat, 1.5–2 cm thick.
12. Flat metal support (1 cm), precooled at −20 °C. The surface should be large enough to accommodate several Petri dishes (to crosslink more samples at the same time), but small enough

to be easily inserted and removed from the available Crosslinker. This support is placed on the top of the freezing pack, to prevent heating of the samples during the incubation/irradiation cycles required for psoralen crosslinking.

2.4 Genomic DNA Extraction (S. cerevisiae) (See Note 2)

1. Spheroplasting buffer: 1 M sorbitol, 100 mM EDTA pH 8.0, 0.1% β-Mercaptoethanol, 100 U/ml Lyticase. This buffer is freshly prepared from the following stocks: 2 M Sorbitol; 0.5 M EDTA pH 8.0; 14.3 M β-Mercaptoethanol (pure liquid); Lyticase (Sigma, cat. L4025) 1000 U/ml in water (store 1 ml aliquots at −20 °C).
2. RNaseA stock solution (10 mg/ml in Tris–HCl pH 7.4) prepared according to [28], freeze aliquots at −20 °C.
3. Proteinase K stock solution (20 mg/ml in water), freeze aliquots at −20 °C.
4. Chloroform/isoamylalcohol 24:1. This solution can be stored at RT in a hood for unlimited time.
5. Kimble glass tubes (KIMBLE HS No. 45500-30).
6. Solution I: 2% w/v CTAB (cetyltrimethylammoniumbromide), 1.4 M NaCl, 100 mM Tris–HCl pH 7.5, 25 mM EDTA pH 8.0 (*see* **Note 3**). Filter Solution I and Solution II to avoid the formation of aggregates during DNA preps. These solutions can be usually stored for 2–3 months at RT. They should be refiltered or freshly prepared if a precipitate is detectable.
7. Solution II: 1% CTAB, 50 mM Tris–HCl pH 7.5, 10 mM EDTA (*see* **Note 3**).
8. Solution III: 1.4 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA.
9. Isopropanol.
10. 70% EtOH, stored at RT.
11. 1× TE buffer.

2.5 Genomic DNA Extraction (Mammalian Cells)

1. Lysis buffer (QIAGEN buffer C1: 1.28 M Sucrose; 40 mM Tris–HCl pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100). Dissolve 483.14 g sucrose, 4.06 g $MgCl_2 \cdot 6H_2O$, and 4.84 g Tris base in 680 ml ddH_2O. Add 42 g Triton X-100 (100%). Adjust the pH to 7.5 with HCl. Adjust the volume to 1 L with ddH_2O. Keep at 4 °C.
2. Digestion buffer (QIAGEN buffer G2: 800 mM Guanidine-HCl; 30 mM Tris–HCl pH 8.0; 30 mM EDTA pH 8.0; 5% Tween-20; 0.5% Triton X-100). Dissolve 76.42 g guanidine HCl, 11.17 g $Na_2EDTA \cdot 2H_2O$, and 3.63 g Tris base in 600 ml of ddH_2O. Add 250 ml 20% Tween-20 solution and 50 ml 10% Triton X-100 solution. Adjust the pH to 8.0 with NaOH. Adjust the volume to 1 L with ddH_2O. Store at RT.

3. Ice-cold ddH_2O.
4. Ice-cold 1× PBS.
5. Proteinase K stock solution 20 mg/ml in ddH_2O (Roche, cat. 03115852001). Store at −20 °C.
6. Chlorophorm/isoamylalcohol 24:1.
7. Isopropanol.
8. Ethanol 70%.
9. 30 ml glass centrifugation tubes, KIMBLE HS No. 45500-30.
10. Eppendorf centrifuge 5810R, rotor A-4-81.
11. Sorvall Evolution RC, rotor HB-6 swinging (8000 rpm = 10,459 × *g*).

2.6 Sperm DNA Extraction (Xenopus Egg Extracts)

1. EB EDTA buffer: 50 mM Hepes-KOH pH −7.5, 100 mM KCl, 2.5 mM $MgCl_2$,1 mM EDTA.
2. Cy3™-dCTP (GE HealthCare cat. PA35021). Dilute 1:50 in EB-EDTA buffer and store in aliquots at −20 °C.
3. EB EDTA sucrose buffer: EB EDTA +30% sucrose.
4. Proteinase K stock solution 20 mg/ml in ddH_2O (Roche cat. 03115852001). Store at −20 °C.
5. Chlorophorm/isoamylalcohol 24:1.
6. Ethanol 70%.
7. 1× TE buffer.

2.7 DNA Digestion and Enrichment of Replication Intermediates

1. RNase A (Ribonuclease A Type I-AS, Sigma-Aldrich, R5503) 10 mg/ml. Store in aliquots at −20 °C.
2. RNase III (ShortCut RNase III, NEB M0245) 10 mg/ml. Store aliquots at −20 °C.
3. QBT equilibration buffer. Dissolve 43.83 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol and 15 ml 10% Triton X-100 solution (v/v). Adjust the volume to 1 L with distilled water.
4. Stock solution 10 mM Tris–HCl pH 8.0, 300 mM NaCl. The solution can be prepared in large volume (50 ml), stored at RT in plastic tubes, and reused for different experiments. 5 M NaCl and 1 M Tris–HCl pH 8.0 stocks are required to prepare this and the following solutions.
5. Stock solution 10 mM Tris–HCl pH 8.0, 900 mM NaCl can be prepared in large volume (50 ml), stored at RT in plastic tubes, and reused for different experiments.
6. Stock solution 10 mM Tris–HCl pH 8.0, 1 M NaCl can be prepared in large volume (50 ml), stored at RT in plastic tubes, and reused for different experiments.

7. Stock solution 10 mM Tris–HCl pH 8.0, 1 M NaCl, 1.8% caffeine (w/v) can be prepared in large volume (50 ml), but requires extensive incubation at 50 °C and stirring or vortexing to help caffeine dissolution. The stock needs to be filtered (syringe filter, 0.2 μM), can be stored at RT in plastic tubes, and can be reused for different experiments during 4–6 months. A new stock should be prepared when a precipitate is detectable.
8. QIAGEN Genomic-tip 20/G (QIAGEN, Cat. No. 10223).
9. Millipore size-exclusion columns: Amicon ultra 100K membrane (Millipore, cat. UFC510096).
10. 1× TE buffer.

2.8 Preparation of Carbon-Coated Grids

1. High Vacuum Evaporator MED 020 (BalTec), with two EK 030 electron guns (C and Pt/C), quartz crystal, thin film monitor QSG 100, and control unit EVM 030 (*see* **Note 4**).
2. Mica Sheets (Plano, mica high grade quality V1, 25 × 76 mm, cat. 56).
3. Wolfram cathodes for electron guns (BALTIC, BP2317).
4. Carbon rods (BALTIC, 3 × 50 mm, cat. BP2217 P = 6).
5. Scotch solution: 20–30 cm of Scotch tape in 100 ml of chloroform in a glass bottle (Fig. 2a). After quick stirring of the bottle, the chloroform turns yellowish, dissolving the tape adhesive (the cellophane support does not dissolve). This solution can be stored at RT for at least 1 year.
6. Supporting Teflon-wire mesh stand (Fig. 2b; *see* **Note 5**).
7. Filter paper circles (Macherey-Nagel, 4310045, diameter: 45 mm).
8. Filter paper circles (Macherey-Nagel, 431009, diameter: 90 mm).
9. EM-grade water (*see* **Note 6**).
10. Copper 3.05 mm grids, 400 mesh (Plano, cat. G2400C; *see* **Note 7**).

2.9 DNA Spreading by the "BAC Method"

1. P2 pipette.
2. EM-grade water (*see* **Note 6**).
3. EtBr stock (10 mg/ml, Sigma, cat. E1510). It can be stored at 4 °C for at least 1 year.
4. EtBr working solution (33.3 μg/ml). It is freshly prepared before each set of spreadings, adding 1 μl of EtBr stock (10 mg/ml) to 300 μl of EM-grade water.
5. Formamide (pure, Sigma-Aldrich, cat. 47680).
6. Glyoxal: 40% solution in water (Merck, cat. 4910).

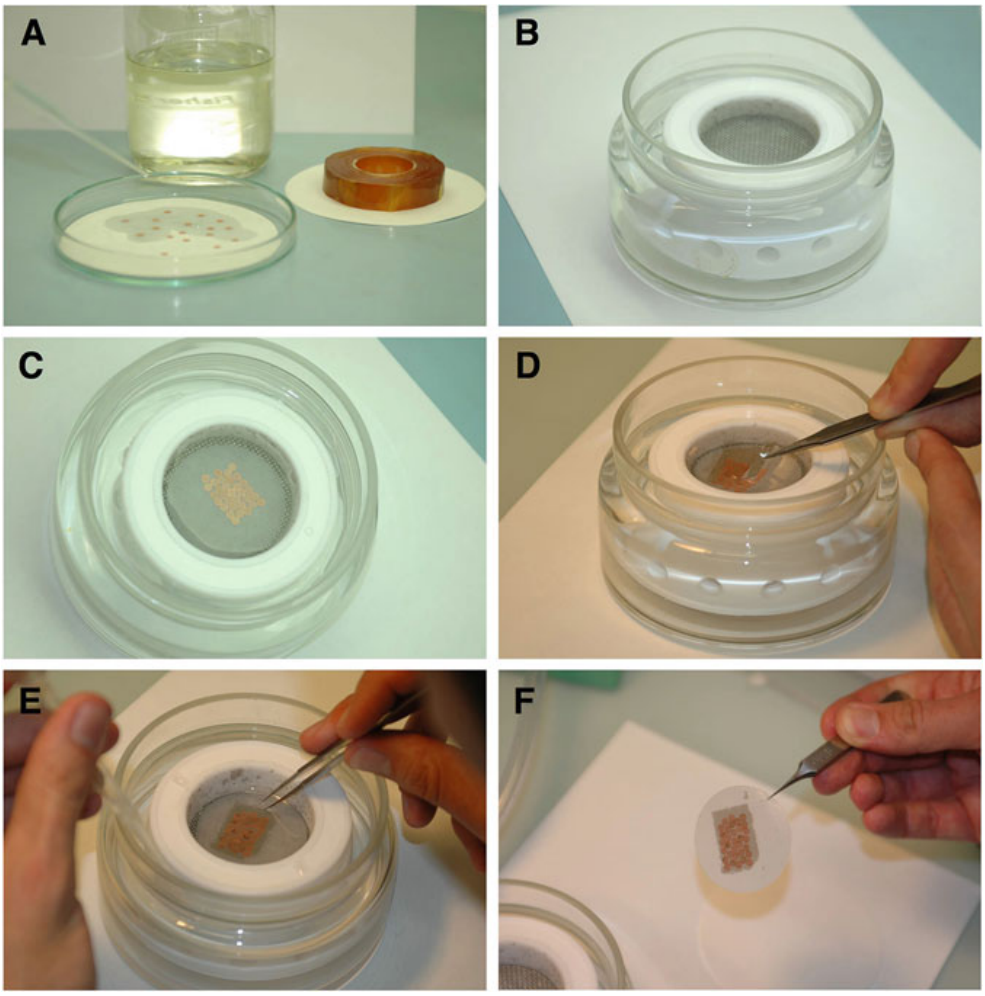

Fig. 2 Series of photographs showing crucial steps in the preparation of carbon-coated grids (*see* Subheading 3.8 for details)

7. BAC stock: BenzyldimethylalkylAmmoniumChloride (Bayer-Leverkusen, n-alkyl mixture: C12H25, 60%; C14H29, 40%; *see* **Note 8**) 0.2% w/v in Formamide. This solution can be stored at RT for at least 1 year.
8. BAC working solution (BAC 1:20): just before each set of spreadings, the BAC stock is diluted 1:20 in 1× TE (20 μl of the diluted stock are prepared in a microfuge tube and are normally sufficient for all spreadings performed during the day).
9. Ethanol 100%, molecular biology grade.
10. Uranyl acetate stock: uranyl acetate (UrAc, Fluka, cat. 73943) 5 mM in HCl 5 mM. This stock solution can be stored at 4 °C for at least 1 year. Uranyl acetate has a typical radioactivity of 0.37–0.51 μCi/g. This mild radioactivity level is not sufficient to be harmful while the material remains external to the body. The use of standard protective clothing (gloves, glasses, lab coat) is therefore sufficient to work safely.

11. Uranyl Acetate working solution (0.5 mM UrAc, 0.5 mM HCl, 90% Ethanol): just before each set of spreadings, the UrAc stock is diluted 1:10 in 100% Ethanol.
12. Round filter paper (Macherey-Nagel, 431009, diameter: 90 mm).
13. Tissue culture dishes, 60 × 15 mm (*see* **Note 9**).
14. Mica sheets (Plano, mica high grade quality V1, 25 × 76 mm, cat. 56).
15. Graphite powder (BalTec, cat. LZ 02096 VN).
16. Fine tweezers with bent points (*see* **Note 10**) or Dumont pinzette (Plano T539).

2.10 Platinum-Carbon Rotary Shadowing

1. High Vacuum Evaporator MED 020 (BalTec; *see* **Note 4**), with two EK 030 electron guns (C and Pt/C), quartz crystal, thin film monitor QSG 100, control unit EVM 030, rocking rotary stage, and specimen table (54 mm) for 20 grids (3.05 mm, Fig. 4a; *see* **Note 11**).
2. Carbon/Platinum rods: Carbon rods 2 × 20 mm (BALTIC BP2260); platinum insets (BALTIC BP2261).
3. Micrometric control of rocking rotary stage angle: the standard knob of the MED020 is substituted by a Precision Rotation Platform PR01 with Adapter Plate PR01A, Thorlabs, Newton, NJ, USA (*see* **Note 12**; Fig. 4c and d, black arrow).

2.11 Visualization at the Transmission Electron Microscope, Contour Length Measures, and Statistics

1. Transmission Electron Microscope, connected to a computer-driven CCD-camera. Minimal resolution required is 1k × 1k if the camera is mounted on the bottom port, while it should preferably reach 2k × 3.5k if mounted on the side 35 mm port.
2. Software for Camera Control and storage of acquired images in original and **.tiff* format (i.e., Digital Micrograph).
3. Software package to produce a composite image (montage) from adjacent images.
4. ImageJ or other software allowing contour length measurements on **.tiff* files.
5. Standard statistic applications (i.e., Microsoft Office Excel).
6. Large hard disk capacity or external hard disk for storage of a high number of large image files (*see* **Note 13**).

3 Methods

3.1 In Vivo Psoralen Crosslinking (S. cerevisiae)

1. Samples are typically collected at different time points during synchronization experiments (*see* **Note 14**). Every sample corresponds to 4×10^9–1×10^{10} cells (400 ml of culture

1–2.5 × 10^7 cells/ml). Spin cells down at 3200 × *g* for 10 min. Resuspend the cells in ice-cold water, transfer the suspension to 50 ml tubes. Spin cells down at 3200 × *g* for 5 min at 4 °C (*see* **Note 15**).

2. Resuspend the pellet in 20 ml of ice-cold water. Transfer cells into an 8.5 cm diameter Petri dish. Several samples can be simultaneously crosslinked with the following procedure.
3. Install the five monochromatic 365 nm lamps in the crosslinker. Make sure that they are all properly inserted and that they all light up when starting the crosslinker.
4. Add 1 ml TMP stock solution (10 μg/ml final concentration) to the cell suspension in the Petri dish. Mix well with a pipette and incubate for 5 min in the dark on the precooled metal support (*see* **Note 16**). Place the precooled metal support with the Petri dishes on the freezing pack. Irradiate the sample for 3 min.
5. Repeat **step 4** once (*see* **Note 17**).
6. Transfer the cell suspension to a 50 ml tube and keep it on ice. Wash the dish twice with 1 ml water to remove all the cells and pool the washes in the same 50 ml tube. Spin down the cells at 3200 × *g* for 10 min. Use the cell pellet for DNA extraction (*see* Subheading 3.4).

3.2 In Vivo Psoralen Crosslinking (Mammalian Cells)

1. Samples can be collected from asynchronously growing cells or at different time points in synchronization experiments (*see* **Note 14**). Every sample corresponds to 2.5–5.0 × 10^6 cells (150 × 20 mm tissue culture dish, 50–80% confluency for U2OS cells). After standard trypsinization (or collection, for cells in suspension), transfer the cells to 15 ml Falcon tubes and spin them down at 600 × *g* for 5 min. Wash the cell pellet once (by resuspension/centrifugation) with 5 ml ice-cold 1× PBS (*see* **Note 15**).
2. Resuspend the pellet in 10 ml ice-cold 1× PBS. Transfer cells into a tissue culture dish 60 × 15 mm. Several samples can be simultaneously crosslinked with the following procedure.
3. Insert the five monochromatic 365 nm lamps in the crosslinker. Make sure that they are all properly inserted and that they all light up when starting the crosslinker.
4. Add 0.5 ml of TMP stock solution (10 μg/ml final concentration) to the cell suspension in the Petri dish. Mix well with a pipette and incubate for 5 min in the dark on the precooled metal surface (*see* **Note 16**). Place the metal support with the Petri dishes on the top of the freezing pack. Irradiate the sample for 3 min.
5. Repeat **step 4** once (*see* **Note 17**).

6. Transfer the suspension back to a 50 ml tube and keep it on ice. Wash the dish twice with 1 ml 1× PBS to remove all cells. Spin down the cells at 600 × *g* for 5 min. Resuspend the cell pellet in 2 ml 1× PBS and proceed with DNA extraction (*see* Subheading 3.5).

3.3 Psoralen Crosslinking of Replicating Sperm DNA (Xenopus Egg Extracts)

1. Samples are typically collected 30–60 min after sperm nuclei addition in the Xenopus extracts. Replicating interphase extracts are prepared as described in [29]. Every sample corresponds to 200–300 μl of extract to which 4000 nuclei/μl are added.
2. Split the sample into 100 μl aliquots in microfuge tubes and incubate at 23 °C.
3. (Optional) To control the timing of replication in the extract, add 5 μl Cy-3 dCTP to 100 μl of the sample (extract + sperm) and check visible incorporation by standard immunofluorescence microscopy (*see* **Note 18**).
4. When 80–90% of the nuclei start incorporating Cy-3 dCTP (ideally 45–50 min after sperm addition), arrest the DNA replication by diluting each 100 μl aliquot with 200 μl of cold EB-EDTA buffer and incubate on ice.
5. Pool the aliquots corresponding to each sample.
6. Underlay the samples with 2 volumes of cold EB-EDTA Sucrose Buffer.
7. Spin at 8600 × *g* at 4 °C for 5 min.
8. Remove the supernatant carefully so as not to disturb the pellet.
9. Resuspend the pellet in 100 μl of ice-cold EB-EDTA using a P200 pipette with cut tips.
10. Transfer the samples to a precooled round-bottom microtiter plate on a precooled metal support.
11. Insert the five monochromatic 365 nm lamp in the crosslinker. Make sure that they are all properly inserted and that they all light up when starting the crosslinker.
12. Add 5 μl of TMP stock solution (10 μg/ml final concentration) to each 100 μl nuclei suspension and mix by pipetting with cut tips. Incubate for 5 min in the dark on a precooled metal support. Place the metal support with the microtiter plate on the top of the freezing pack and irradiate the sample for 3 min.
13. Repeat **step 12** two more times.
14. Recover the nuclei suspension from the wells into microfuge tubes. Centrifuge at 5000 × *g* for 5 min and resuspend nuclei in 300 μl of EB-EDTA buffer using a precut 1 ml tip. Proceed with DNA extraction (*see* Subheading 3.6).

3.4 Genomic DNA Extraction by CTAB Method (S. cerevisiae) (See Note 2)

1. Resuspend the cells in 5 ml of Spheroplasting buffer. Incubate at 30 °C for 45 min. Invert the tube several times during incubation. Spin down the spheroplasts at 6000 × *g* for 10 min.
2. Resuspend the spheroplasts in 2 ml of distilled water and rapidly add 2.5 ml of Solution I, 200 µl of RNaseA (10 mg/ml). Incubate for 30 min at 50 °C, then add 200 µl of Proteinase K (20 mg/ml). Gently mix the sample.
3. Incubate for 1.5–2 h at 50 °C. Invert the tube several times during incubation. Note that the solution has to become clear (no clumps!); if necessary, use precut 1 ml tips to break clumps. If clumps are still present after 2 h, add 100 µl of Proteinase K (20 mg/ml) and incubate O.N. at 30 °C.
4. Centrifuge for 10 min at 3200 × *g*: keep the pellet (cellular debris) for further extraction (*see* Subheading 3.4, **steps 9–11**). Transfer the supernatant carefully to a 15 ml tube containing 2.5 ml of chloroform/isoamylalcohol 24:1 at RT.
5. Mix by inverting the tubes several times and spin at RT at 3200 × *g* for 10 min. Note that a white protein layer is formed between the two phases. Carefully remove the clear upper phase and transfer it to a 30 ml Kimble glass tube.
6. Gently add 10 ml (2 volumes) of Solution II, cover with parafilm and invert several times. The solution should slowly become turbid. If necessary, incubate for 10–15 min at RT until some turbidity is detectable.
7. Centrifuge for 10 min at 12,000 × *g* in a proper swinging bucket rotor and discard the supernatant.
8. Add 2 ml of Solution III and incubate the solution briefly at 37 °C until the pellet is completely dissolved.
9. Resuspend the pellet from **step 4** in 2 ml of Solution III, mix vigorously, and incubate at 50 °C for 1 h (use precut 200 µl tips to help complete resuspension). Check that the solution is finally homogeneous.
10. Transfer the solution carefully to a 15 ml tube containing 1 ml of chloroform/isoamylalcohol 24:1 at RT.
11. Mix by inverting the tubes several times and spin at RT at 3200 × *g* for 10 min. Note that a white protein layer is formed between the two phases. Carefully remove the clear upper phase (ca. 2 ml) and pool it in the Kimble glass tube from **step 8** (4 ml final volume).
12. Precipitate the DNA by adding 4 ml of isopropanol (RT). Gently mix the sample to ensure a proper DNA precipitation. Spin down the DNA at 12,000 × *g* for 10 min in a swinging bucket rotor.
13. Discard the supernatant and wash the pellet with 1 ml of Ethanol 70% (RT).

14. Remove as much ethanol as possible and briefly spin the pellet to accumulate residual ethanol at the bottom of the tube. Remove it using a 200 µl pipette. Further dry the pellet by briefly incubating the open Kimble glass tube in a 37 °C water bath.
15. Add 200 µl of 1× TE to the dried pellet. Cover the Kimble glass tube with parafilm and incubate for 30 min at 37 °C (or O/N at RT) to ensure proper resuspension of the DNA pellet. Spin briefly and transfer the DNA solution to a microfuge tube using a precut 200 µl tip, to avoid shearing of genomic DNA.
16. Check the quality and the concentration of DNA preps, as well as possible RNA contamination (*see* **Note 21**) by agarose-gel electrophoresis. The standard yield, starting with 4×10^9–1×10^{10} cells, is therefore 10–60 µg of genomic DNA from each sample.

3.5 Genomic DNA Extraction (Mammalian Cells)

1. Add to the cell suspension in 1× PBS (Subheading 3.2, **step 6**) 2 ml of ice-cold lysis buffer and 6 ml of ice-cold ddH_2O.
2. Mix by inverting the tube several times and incubate on ice for 10 min.
3. Spin the lysed cells at 4 °C for 15 min at 1300 × *g*. Discard the supernatant.
4. Add 1 ml of ice-cold lysis buffer and 3 ml of ice-cold ddH_2O. Resuspend the pelleted nuclei completely by vortexing.
5. Spin the lysed cells at 4 °C for 15 min at 1300 × *g*. Discard the supernatant completely.
6. Add 100 µl of ice-cold PBS and resuspend the nuclei with a cut 200 µl tip. Make sure that no clumps of cells are left.
7. Add 5 ml of digestion buffer. It is crucial NOT to VORTEX or rotate the tube to avoid clumping of the cells in the center of the tube. Add 200 µl of proteinase K stock and incubate at 50 °C until the solution is clear (1–2 h).
8. Let the sample cool down to RT, then transfer it into a 50 ml tube containing 5 ml of chlorophorm/isoamylalcohol. Close the tube properly and invert it vigorously but carefully 30 times (solution turns milky). Immediately pour sample into a glass centrifugation tube.
9. Centrifuge at 4 °C for 20 min at 10,500 × *g* (phase separation).
10. Carefully transfer upper phase into a new glass tube using a cut 1 ml tip. Add the same volume of isopropanol and mix well to precipitate the DNA.
11. Centrifuge at 4 °C for 10 min at 10500 × *g*.
12. Wash DNA with 70% ethanol. Spin at 4 °C for 5 min at 10,500 × *g*.

13. Air dry the pellet and resuspend in 1× TE buffer (200–400 µl). Pay attention not to overdry DNA pellet to prevent irreversible "agglutination" of DNA molecules. The standard yield is 10–50 µg of genomic DNA for 2.5–5.0 × 10^6 cells.

3.6 DNA Extraction from Sperm Nuclei (Xenopus Egg Extracts)

1. Add to the crosslinked nuclei (Subheading 3.3, **step 14**) 1.5 µg/ml final concentration of proteinase K and incubate for 2 h at 50 °C.
2. Add equal volume of phenol:chloroform:isoamylalcohol—25:24:1 to the above solution and shake vigorously. Spin at 18,500 × *g* for 10 min.
3. Transfer the supernatant to a new microfuge with a cut 1 ml tip.
4. Add equal volume of 100% isopropanol to the supernatant to precipitate the DNA. Incubate at 4 °C for 10 min. Spin the sample at 18,500 × *g* for 10 min at 4 °C. Discard the supernatant.
5. Wash pellet with 500 µl of 70% ethanol and spin down the sample for 5 min.
6. Discard the supernatant and briefly centrifuge at 1700 × *g*. Remove the residual ethanol carefully with a 20 µl pipette.
7. Incubate the tube at 37 °C to evaporate the excess ethanol.
8. Resuspend the pellet in 100 µl TE.
9. Assess quality and quantity of the DNA by UV spectrophotometry and by gel electrophoresis.

3.7 DNA Digestion and Enrichment of Replication Intermediates (See Note 19)

1. 10–15 µg (yeast, mammalian cells) or 5 µg of DNA (Xenopus) is digested with 50–100 U of restriction enzyme in the proper buffer for 3–5 h at 37 °C. Standard restriction enzymes used are PvuI for *S. cerevisiae* genomic DNA, PvuII HF for mammalian genomic DNA, and NdeI for Xenopus sperm DNA (*see* **Note 20**). The volume of the restriction reaction is normally set to 250 µl, but can be increased to account for more diluted DNA preps (the reaction mix is anyway diluted in **step 5** in Subheading 3.7). If necessary, add small amounts of the proper RNAse enzymes (*see* **Note 21**).
2. Equilibrate QIAGEN-tip 20 by applying 1 ml Buffer QBT. Allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of the detergent in the equilibration buffer (Triton X-100).
3. Wash the QIAGEN-tip 20 column three times with 1 ml of 10 mM Tris–HCl pH 8, 1 M NaCl.
4. Equilibrate the column six times with 1 ml of 10 mM Tris–HCl pH 8, 300 mM NaCl.

5. After the required incubation time (3–5 h, *see* **step 1**), adjust the digestion mix to 300 mM NaCl final concentration, by adding 5 M NaCl stock (check the restriction buffer composition). Adjust the final volume to 600 µl with 10 mM Tris–HCl pH 8, 300 mM NaCl.
6. Apply the pre-equilibrated digestion mix and allow it to enter the resin by gravity-flow.
7. Wash the QIAGEN-tip 20 with two times 1 ml 10 mM Tris–HCl pH 8, 850 mM NaCl. Collect the flow-through in 2 ml tube and save for an analytical gel.
8. Add to the column 600 µl of 10 mM Tris–HCl pH 8, 1 M NaCl, 1.8% caffeine at 50 °C. Collect the flow-through, enriched in RIs in 1.5 ml tube.
9. Purify and concentrate the DNA (removing residual RNA, small linear fragments, and microscopic dirty particles) using an Amicon size-exclusion column.

   Load the 600 µl of elution from **step 8** into Amicon column. Spin the column for 8 min at 9000 rcf.
10. Wash the membrane with 200 µl of 1× TE and spin the column for 5 min at 9000 rcf. Wash again with 200 µl of 1× TE and spin the column for 4 min at 8000 rcf until 15–30 µl remains.
11. Invert the Amicon filter and short spin into a fresh Amicon tube.

    Note: Transfer the sample to a fresh 1.5 ml eppendorf tube to prevent evaporation of the sample solution.
12. Load a 1 µl aliquot on an agarose gel to check DNA quality and concentration. If necessary, adjust the final volume (by adding 1× TE or concentrating the sample in a standard vacuum evaporator) to reach the optimal DNA concentration of 10–50 ng/µl (*see* **Note 22**).

3.8 Preparation of Carbon-Coated Grids

1. Cleave a 2 cm × 2 cm sheet of mica and place it (with the freshly cleaved surface facing up) on the support plate of the MED 020, at a distance of about 12 cm to the carbon evaporator gun. Place the quartz sensor as close as possible to the mica. Cover the mica with the tilting shutter, properly position the glass vacuum chamber wall, and start the turbomolecular pump of the MED020.
2. At a vacuum of about 3×10^{-5} mbar, preheat the filament of the carbon electron gun. Adjust voltage and current applied to the electron gun to reach a constant evaporation rate of 0.03–0.05 nm/s (detected on the QSG 100 thin film monitor). Open the tilting shutter and start measuring the carbon film thickness on the QSG 100 (*see* **Note 23**). When the thickness readout is 3.5–4.5 nm (50–70 Hz), close the shutter

and shut off the electron gun. Generally, 2–3 carbon films can be produced in series (*see* **Note 24**).

3. Remove the carbon-coated mica sheet from the MED 020. Carbon-coated mica sheets can be stored at this stage up to 4–5 weeks.
4. When ready to transfer the carbon film on the grids, place the carbon-coated mica sheet (carbon side up) in a Petri dish on wet filter paper and incubate it at 37–42 °C for 30 min–1 h.
5. Spread copper grids on filter paper in a glass Petri dish and make sure that the glossy side of all grids is facing up. 30–35 grids should be used for each 2 cm × 2 cm carbon-coated mica sheet. Place the Petri dish in a hood and, using a Pasteur pipette, rinse each grid with one drop of Scotch solution (Fig. 2a). Air-dry the grids. Repeat the procedure two to three times to make sure that all grids have been extensively rinsed in Scotch solution. The tape adhesive will keep the carbon film attached.
6. Fill the supporting wire mesh stand with EM-grade water (Fig. 2b). A round filter paper (diameter: 45 mm) is submerged in water and 30–35 grids (glossy side up) are placed on its surface in a close and ordered distribution (Fig. 2c).
7. The carbon-coated mica sheet is then removed from the wet filter paper, briefly dried on its lower side (no carbon) by a napkin, and slowly lowered into the water (carbon side up) at an angle of approximately 45° (Fig. 2d), until the carbon film is completely released and floating on the water surface (*see* **Note 25**). Discard the mica support.
8. The carbon film is finally placed on the grids by carefully lowering the water level in the supporting Teflon-wire mesh stand, using an aspirator connected to a vacuum pump. Use tweezers to correctly position the carbon film on the grids, while lowering the water level (Fig. 2e).
9. Once the water has been removed, take the filter paper with the carbon-coated grids (Fig. 2f), cut off the excess of wet filter paper around the grids, and let the carbon-coated grids dry for at least one night before using them for DNA spreading experiments. Although some variability has been observed, carbon-coated grids are usually reliable for DNA absorption for about 4–5 weeks after carbon-film production.

3.9 DNA Spreading for EM Visualization

3.9.1 "Native" DNA Spreading by the "BAC Method"

1. For each DNA spreading, distribute up to eight droplets (10–15 μl) of EtBr working solution on a piece of parafilm. Carefully place a carbon-coated grid on the top of each drop, with the carbon-side facing the liquid (Fig. 3a; *see* **Note 26**). Incubate grids for 20–45 min. Prevent evaporation by covering the parafilm with the lid of the 15 cm Petri dish. Just before

spreading the sample on the hypophase (Subheading 3.9.1, **step 6**), take each grid, remove the excess of EtBr solution contacting the filter paper, and place the dried grids (carbon side down) on the top of filter paper (Fig. 3b).

2. Using a P2 pipette, mix at the very bottom of a 1.5 ml-microfuge tube: 1 μl of Formamide, 0.4 μl of BAC 1:20 (*see* **Note 27**).

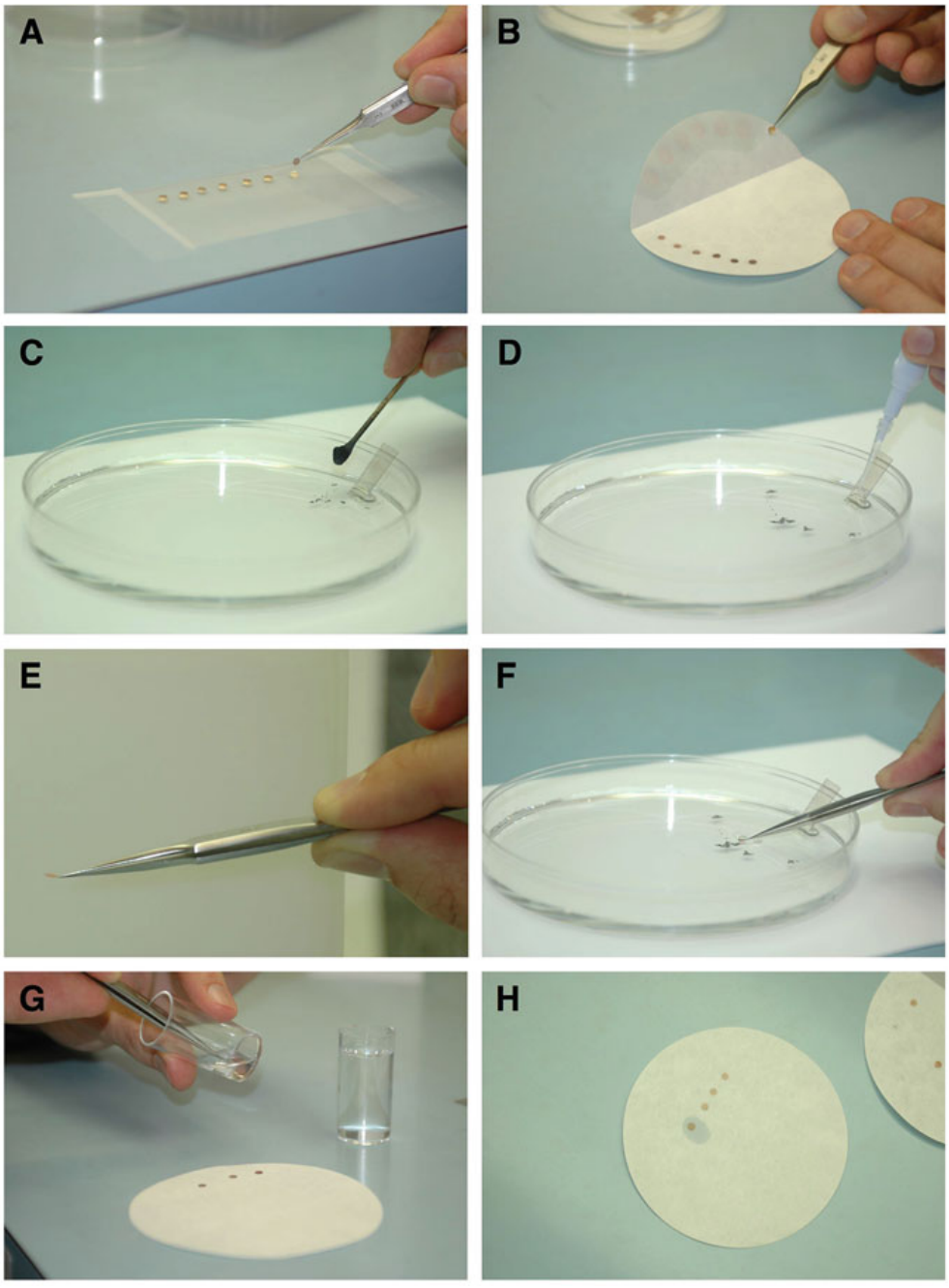

Fig. 3 Series of photographs showing crucial steps in the BAC-DNA spreading method (*see* Subheading 3.9 for details)

3. In a separate drop on the side of the same tube (*see* **Note 28**), mix: 1.5–3 ng of plasmid DNA of known size (3–10 kb; internal size maker) and 10–50 ng of sample DNA (genomic DNA enriched for Replication Intermediates, *see* Subheading 3.7, **step 12** and **Note 29**). The total volume of the DNA drop should be 1.25 μl; usually it consists of 0.25 μl plasmid DNA (5 ng/μl) and 1.0 μl sample DNA. Smaller sample volumes can be filled up with 1× TE buffer (*see* **Note 29**). In case of very low DNA concentration in the sample, up to 4 μl of the DNA sample can be added to the spreading. In this case an equal volume of Formamide should be added to the mix, while volumes of plasmid DNA and BAC remain unchanged
4. Pour approximately 20 ml (the minimum volume to cover the surface completely) of EM-grade water in the 15 cm Petri dish: this is called the hypophase. Cleave a mica sheet (about 1 cm × 2 cm) and place it in the water as a ramp, with the freshly cleaved surface facing up (Fig. 3c).
5. Spin the tube containing the sample for a few seconds in a microfuge and aspirate it completely in a 10 μl tip. With a cotton swab sprinkle a few grains of graphite powder (no graphite flakes!) onto the water surface, in close proximity to the mica ramp (Fig. 3c).
6. Pipette the entire sample volume out of the tip and let the droplet touch the ramp few millimeters above the water surface. The BAC-containing drop will immediately slide down the ramp and spread over the water hypophase. The graphite powder will mark the border of the monomolecular detergent film containing the DNA molecules (Fig. 3d; *see* **Note 30**).
7. Using fine tweezers take one carbon-coated, EtBr-treated grid from the filter paper (carbon-side down, Fig. 3e) and pick up part of the DNA film touching the spreading surface in proximity to the graphite powder (*see* **Note 31**). Hold the tweezers to ensure full parallel contact of grid and surface (*see* **Note 10**). Enough pressure should be applied to ensure full contact between the carbon and the DNA-containing film (Fig. 3f). The grid is then removed from the surface and incubated for 15 s for staining in 1 ml of uranyl acetate working solution (in a flat-bottom 20 ml tube; Fig. 3g). After a brief wash (1–2 s) in 100% Ethanol, air-dry the grid (carbon-side up) on filter paper (Fig. 3h) and carefully wipe the tweezers with a napkin (*see* **Note 32**).
8. **Step 7** is repeated for the other grids, ensuring to collect DNA from different regions of the BAC film containing the DNA molecules (*see* **Note 33**).

3.9.2 Denaturing DNA Spreading by the "BAC Method"

To obtain information about in vivo-nucleosome positioning on the RIs (*see* Subheading 1), DNA samples can be denatured just before the BAC spreading. In this case, the presence of the denaturing agents (Formamide and Glyoxal) in the spreading mix is coupled with a short incubation at 42 °C, leading to DNA strand separation at each of the not-crosslinked regions (nucleosomal DNA; Figs. 1 and 8; *see* **Note 34**).

The spreading procedure is identical to the one described above (Subheading 3.9.1), with the exception of **steps 2** and **3**, performed as follows:

2. Using a P2 pipette, mix in a 1.5 ml-microfuge tube: 1.0 μl of Formamide, 0.2 μl of Glyoxal, and 1.0 μl of DNA sample (10–50 ng) from Subheading 3.7, **step 12**. Incubate for 10 min at 42 °C in a water bath and chill immediately after in ice-water.
3. Spin briefly the sample and add: 0.25 μl of plasmid DNA 5 ng/μl (internal size marker, *see* **Note 35**) and 0.4 μl of BAC 1:10. Immediately proceed with **steps 4–8** of the BAC spreading.

3.10 Platinum-Carbon Rotary Shadowing

1. Place the grids on the specimen table, taking care that they are properly fixed (by clips or magnetic stripes) and flat (Fig. 4a). Position the specimen table on the rocking rotary stage and the quartz sensor as close as possible to the specimen table (Fig. 4b).
2. Tilt the rocking rotary stage using the Precision Rotation Platform PR01, so that the angle between the specimen table and the Pt/C gun is exactly and reproducibly 3° (Fig. 4c). The fine micrometric scale on the Rotation Platform enables the reproducibility of the angle (Fig. 4c and d, black arrow). Cover the rotary stage with the tilting shutter, properly position the glass vacuum chamber wall, and start the turbomolecular pump of the MED 020.
3. At a vacuum of 3×10^{-5} mbar (or higher vacuum), preheat the filament of the Pt/C electron gun. Adjust voltage and current applied to the electron gun to reach a constant platinum/carbon evaporation rate of 0.03–0.05 nm/s (*see* **Note 36**), detected on the QSG 100 thin film monitor (*see* **Note 23**). Open the tilting shutter and start the measure of platinum film thickness on the QSG 100. When the detected thickness is 0.4 nm, start the rotation of the specimen table at the minimum speed (about 20 rpm). During the evaporation time (4–5 min) keep adjusting voltage and current applied to the electron gun to maintain the platinum/carbon evaporation rate constant. When the thickness readout at the QSG 100 is 8–10 nm (1800–2000 Hz), close the shutter, stop rotation, and shut off the electron gun. After the gun is cooled down, start the machine again and evaporate another 4–6 nm platinum on your grids while rotating (*see* **Note 37**).

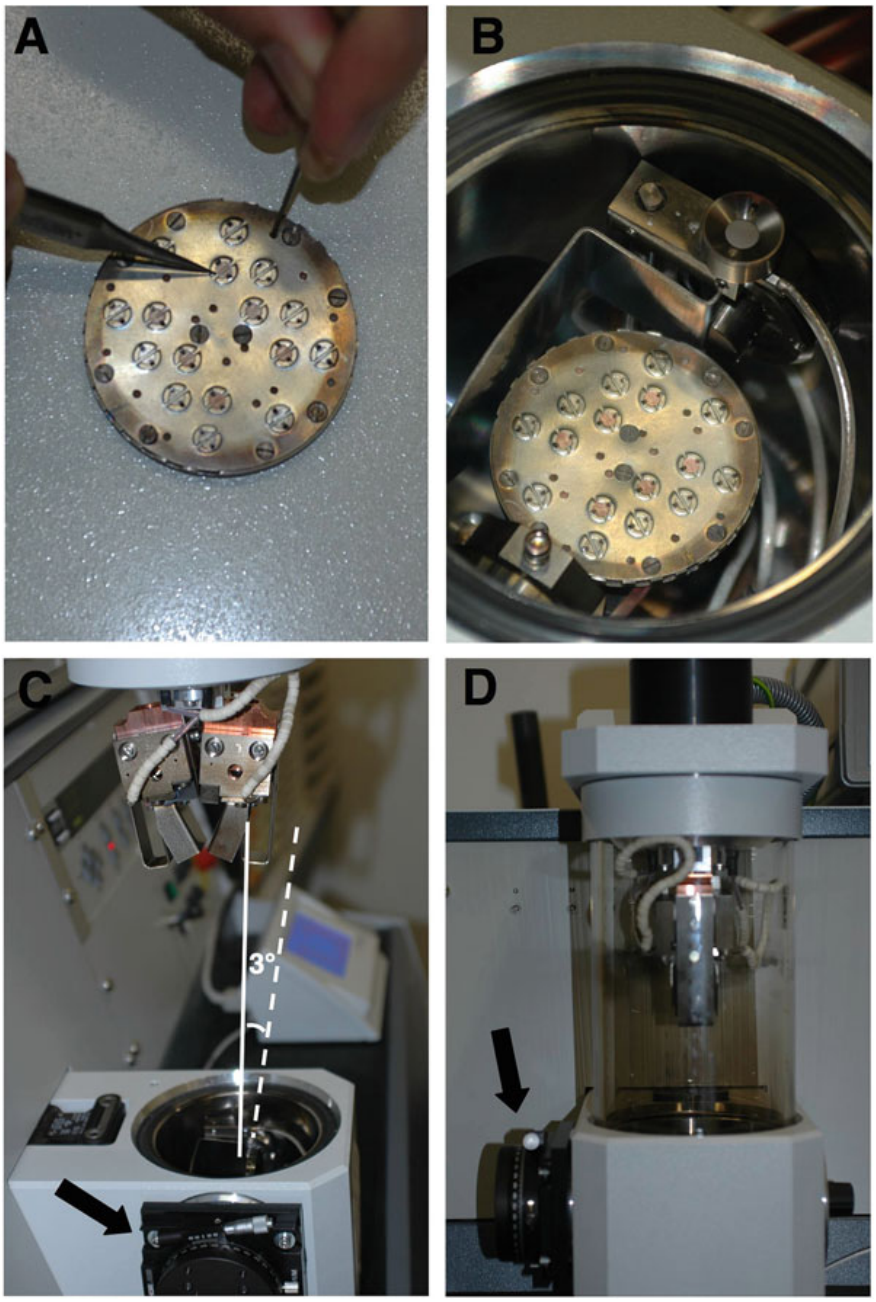

Fig. 4 Series of photographs showing crucial steps in the platinum/carbon rotatory shadowing procedure (*see* Subheading 3.10 for details)

4. Remove the grids from the specimen table and store them properly for transportation. The grids can immediately be analyzed at the Transmission Electron Microscope or can be (re-) analyzed after unlimited storage periods.

3.11 Visualization at the Transmission Electron Microscope, Contour Length Measures, and Statistics

1. The grids can be analyzed at any Transmission Electron Microscope. Duplex DNA in this technique is expected to appear as a 10 nm thick fiber, while ssDNA thickness should be 5–7 nm [20]. Both molecules should be clearly detectable on the homogenously granular background given by the platinum grains deposited on the carbon film (*see* **Note 12**).

2. The plasmid DNA molecules added to the spreading mix can be used as an internal control of DNA absorption by the carbon film and as an internal size marker. Moreover, these circular molecules are often partially denatured (even in "native spreadings") by the short incubation with Formamide and Glyoxal: their partial denaturation offers an easy opportunity to verify whether ssDNA and dsDNA in the same molecule are easily distinguishable by their thickness. At this step, if the concentration of the DNA, the quality of the shadowing, and/or the generally clean appearance of the grid background are not fully satisfactory, the experiment should be repeated, starting from the DNA spreading (or from earlier steps in the sample preparation, in case of recurrent problems, probably resulting from the DNA sample itself).
3. The extensive analysis of a satisfactory sample is usually performed at 5000–20,000×. Despite the enrichment procedure described in Subheading 3.7, linear duplex DNA represents at least 90% of the genomic DNA visualized on the grids (it is retained on the QIAGEN-tip 20 probably because of local "breathing" of the DNA duplex and exposure of ssDNA). The identification of a replication fork requires the recognition of a "3 leg-junction," i.e., a contact point from which three different DNA fibers depart (Fig. 5). These junctions need to be carefully analyzed at higher magnification (50,000–250,000×) to distinguish them from occasional overlaps of two linear molecules in proximity to one of the ends ("4-leg junctions"). At higher magnifications, some ssDNA is usually detectable at one or two sides of the junction, reinforcing the interpretation of the junction as a bona fide replication fork and supporting the identification of the daughter strands (Fig. 5). Furthermore, at least two of the three legs should be equal in length (as the genomic DNA has been digested, the elongation point should be equally distant from the two newly replicated restriction sites). The estimation of DNA molecule length is often complicated by the convoluted distribution of the fibers; this often requires the detailed analysis to be performed only later, once a digital file has been generated. Technical problems, such as partial restriction digestion or breakage of DNA molecules during sample preparation, account for the fraction of replication forks with all three legs different in length, i.e., asymmetric replicated duplexes. In standard conditions these should not represent more than 25–30% of the total replicating molecules, but the proportion of asymmetric forks can increase in conditions of DNA damage, associated with ssDNA accumulation.
4. Although photo-documentation can be obtained on any TEM generating photographic negatives (followed by developing

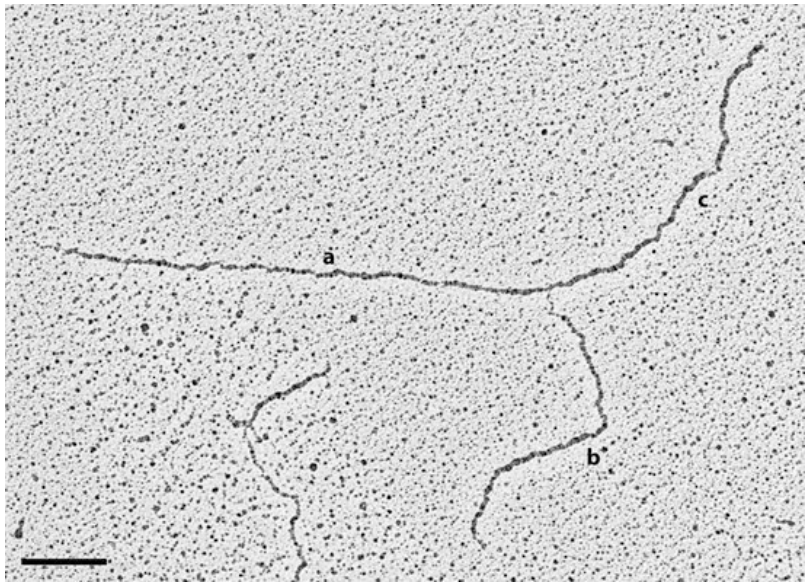

Fig. 5 Normal replication fork visualized by in vivo psoralen crosslinking and TEM. Replicated duplexes (b, c) are of equal length. A ssDNA stretch is clearly visible in proximity to the fork on one of the two replicated duplexes (presumably the lagging strand)

and printing of the corresponding micrographs), the use of a microscope connected to a CCD camera is highly recommended: besides the reduction of the time required for photo-documentation, this procedure allows us to obtain digital files that can be promptly used for contour length measures through Imaging applications (*see* Subheading 3.11, **step 6**).

5. As a general rule, 70–100 replicating molecules should be collected in each experiment to obtain reliable data from the following statistical analysis. Observed trends need to be reproduced in two-three biological replicates. Should the observed differences in the frequency of specific intermediates be small and/or not fully reproducible, statistical assessment on data from three independent experiments is essential.
6. Once the digital files are converted in **.tiff* files, contour length measurements can be performed by standard image analysis applications. ImageJ has been successfully used, but other, more specific applications are being tested and could be more appropriate (and time-saving) for filament recognition and analysis. Once the measurements are performed, data can be analyzed by using standard statistical/graphical applications such as Microsoft Excel or Graphpad Prism.

3.12 Interpretation of Four-Way Junctions

Our most recent studies have identified reversed replication forks (Figs. 6a, b and 7) as frequent replication intermediates under certain conditions, i.e., upon genetic or pharmacological replication interference [11–17, 30]. Importantly, the regressed arm can either be connected to both or only one of the daughter duplexes,

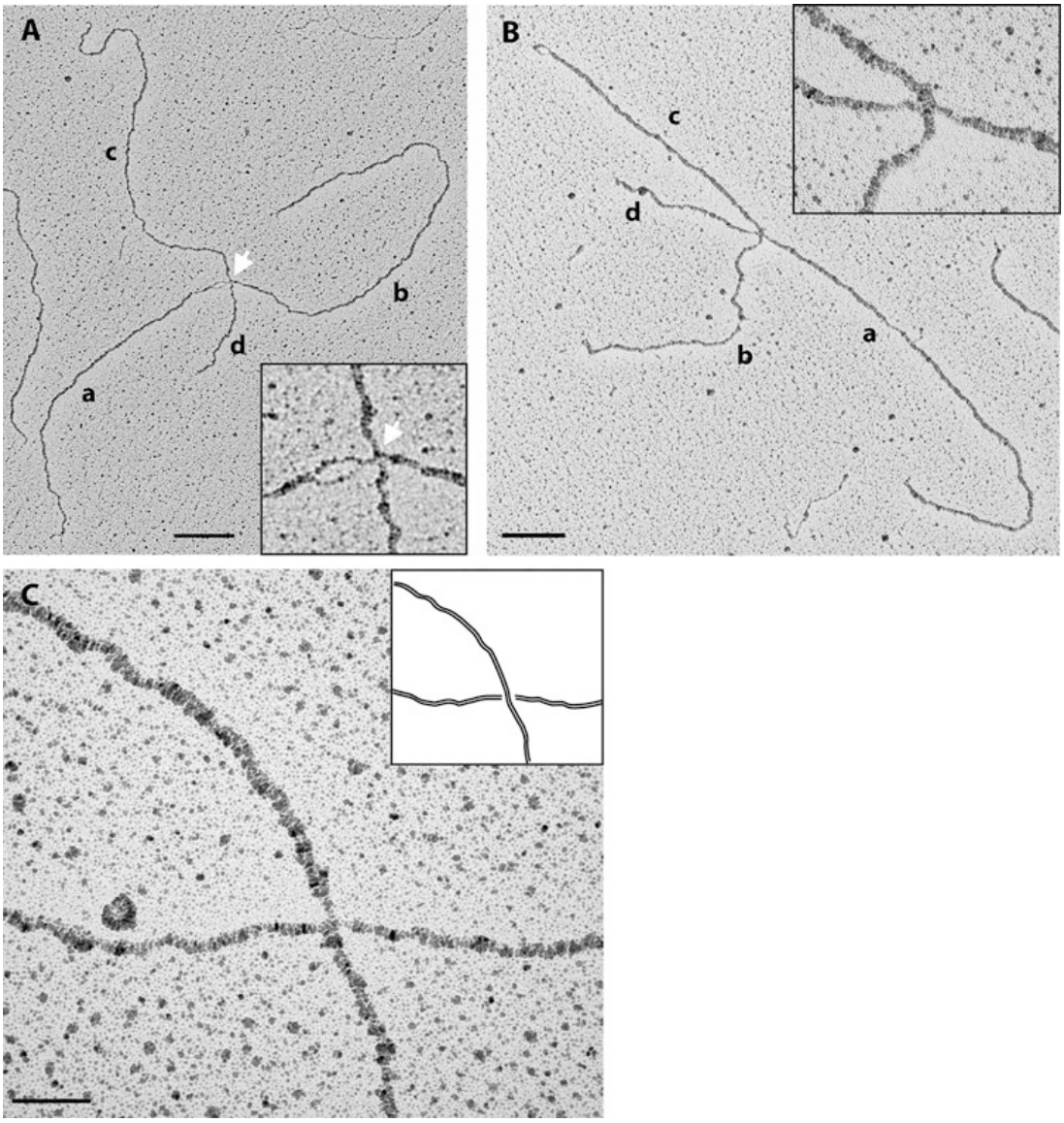

Fig. 6 Compilation of three molecules to assist the identification of reversed forks. (**a**) and (**b**) are representative examples of reversed forks, while (**c**) shows the accidental crossing of two independent DNA molecules (*see* Subheading 3.12 for details)

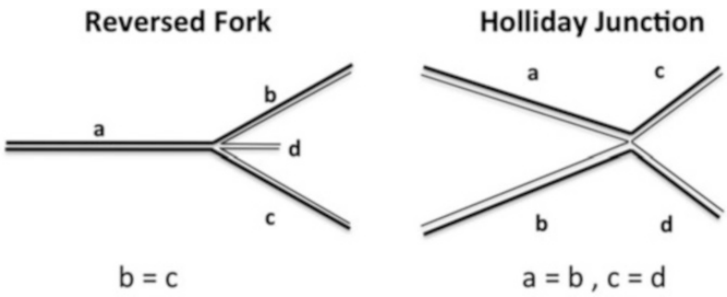

Fig. 7 Drawing on reversed fork vs Holliday junction and their expected features in terms of contour length measurements

and it can be entirely or partially single-stranded. The unambiguous identification of these replication intermediates is based on contour length measurements in combination with several additional criteria. As reversed replication forks are four-way structures, accidental crossings of linear DNA molecules can easily be mistaken for reversed forks. The incidence of random crossings increases with the DNA concentration on the grids; therefore, it is necessary to work with samples with low DNA density to minimize the risk of misinterpretations.

The most important criterion for the assignment of a four-way structure as a reversed fork is the junction itself. If the junction is opened up and allows the clear identification of a romboid structure, the assignment as a reversed replication fork is usually straightforward (Fig. 6a). However, most reversed replication forks display a collapsed junction with little or no opening (Fig. 6b) and therefore require additional parameters for their interpretation. These parameters are the appearance of the junction, and the orientation and the length of the arms.

For a careful interpretation of a four-way junction, taking a high magnification image (135,000–250,000×) of the junction is recommended. All the arms of a reversed fork are connected to each other; therefore, they give the junction a "flat" appearance in one focal plane (Fig. 6b, inset). In contrast, in accidental crossings of linear DNA, one molecule is on the top of the other, resulting in a difference in focal plane that can help to discriminate DNA crossings from true DNA junctions already while taking pictures (Fig. 6c). Moreover, crossings are usually associated with a continuous shadowing of the molecule on the top and a discontinuous platinum deposition on the bottom molecule (Fig. 6c).

As in an unperturbed replication fork, the length of the two new replicated duplexes of a reversed fork should be identical ($b = c$; Fig. 7). In contrast, as typical for all replication intermediates, the length of the parental arm (a) is not defined, as is the length of the regressed (d) arm ($a \neq b = c \neq d$; Fig. 7). Conversely, Holliday junctions connect symmetrically two homologous fragments and will have by definition arms of equal length two by two ($a = b$, $c = d$). Only in those rare cases in which a restriction site on the parental DNA is located in close proximity to the elongation point (late replication intermediate), the same restriction site will also be present on the regressed arm, giving rise to ($a = d$; $b = c$) molecules. The appearance of such molecules would be identical to a late termination intermediate or to a Holliday Junction; therefore, they cannot be unambiguously assigned and should not be considered in the analysis.

The final criterion for the identification of reversed forks is the orientation of the arms of the molecule. Reversed forks and other DNA junctions usually display different orientations of at least three of the four arms of the molecule, whereas the "arms" in

accidental crossings are often aligned along only two axes, sometimes even in a perpendicular fashion (compare Fig. 6b and c).

As for all other replication intermediates (*see* Subheading 3.11, **step 3**), partial digestion and DNA breakage can lead to reversed forks displaying no obvious symmetry in the length of the four arms. Particular attention should be paid to the analysis of the junction at high magnification (*see* above) to assign these molecules as reversed forks. As a general rule, the fraction of asymmetric reversed forks should never exceed the frequency of asymmetry observed in the same samples for the population of "normal" (three-way) replication forks.

3.13 Analysis of Replicating Chromatin Under Denaturing Conditions

Denaturing spreadings (Subheading 3.9.2) allow the analysis of nucleosome density on DNA. For this kind of analysis, a sufficient degree of psoralen crosslinking is required. 80–90% of the linkers should contain at least one crosslink, so that 80–90% of detected ssDNA bubbles (Figs. 1 and 8) represent mononucleosomes of 150–200 bp [2, 22–24]. Nucleosome density is usually expressed by the so-called *r*-value, which is calculated as the combined contour length of all nucleosome bubbles in a given stretch of DNA (Fig. 8a, white), divided by the overall contour length of the same DNA stretch (Fig. 8b, black). A reduced *r*-value indicates a reduction in nucleosome density.

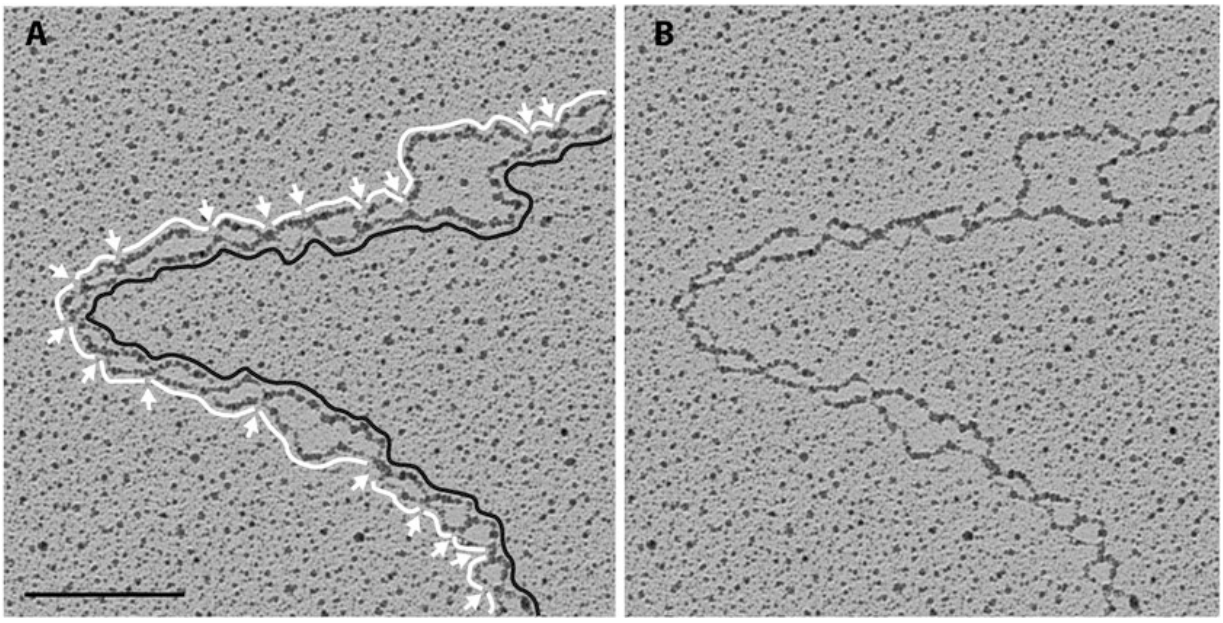

Fig. 8 Example of denaturing molecule (**b**) and system to measure it (**a**). The portion of the molecules organized in nucleosomes is calculated by adding all DNA stretches in the visible ssDNA bubbles (*white tracts*), thus excluding gaps of crosslinked DNA (*white arrows*). This combined length is then divided by the total length of the DNA tract analyzed (*black line*), giving rise to the "*r*-value" (*see* Subheading 3.13 for details)

4 Notes

1. A high efficiency of psoralen crosslinking can be obtained with shorter irradiation times, using monochromatic 365 nm Hg lamps [25]. However, with proper tuning of the irradiation time, this protocol has the advantage of using standard equipment (Biolinker) frequently available in molecular biology laboratories.
2. High quality genomic DNA for EM analysis has also been obtained reproducibly using QIAGEN Genomic-tip 20/G, following the manufacturer's instruction (vortexing steps are substituted by more gentle inversion of the tubes, to avoid shearing of genomic DNA). Nevertheless, the procedure described in detail is the one used for most of the EM data recently published on yeast replicating genomic DNA [5, 8, 31].
3. Help CTAB dissolution by heating the solutions to about 50 °C (do not overheat or boil!).
4. A number of different evaporators are available, with relevant differences in terms of vacuum chamber design and orientation of the electron guns in respect to the specimen. All evaporators can be used to produce carbon films and most of them can be adapted to perform extremely flat angle Pt/C rotary shadowing. The procedure described here allows us to perform Carbon evaporation (Subheading 3.8) and Pt/C rotary shadowing (Subheading 3.10) with the same machine, currently available on the market, with minor technical modifications to the apparatus (*see* **Note 12**). In our experience, the correct maintenance of the apparatus and the constant use/order of identical consumable parts (Wolfram cathodes, carbon rods, platinum insets) is of crucial importance to avoid unexpected problems during evaporation and to obtain reproducibly good carbon layers and rotary shadowing.
5. Supporting wire mesh stands, similar to the one shown in Fig. 2b, can be produced in any workshop. It is composed of two Teflon rings, the lower of which has holes that allow controlled water flow out of the central cavity. A wire mesh stand is placed in-between the two Teflon rings and can accommodate the round filter paper where the grids are placed. Teflon can be substituted by different materials (Plexiglas, plastic, etc.). However, Teflon offers the best resistance to acid washes of the apparatus, occasionally required to remove traces of the carbon films.
6. Excellent results have been obtained with re-distilled water. More recently, standard MilliQ water (produced by a conventional Millipore apparatus), with resistivity of about 18 MΩ cm and total organic content of less than eight parts per billion, has provided satisfactory results. This water is stored in a proper

flask solely dedicated to the EM work, which is directly filled at the MilliQ water apparatus.

7. Nickel grids can be used as an alternative to copper grids to make use of magnetic holders for the evaporation machine. This enables the use of for example the Leica BAF060 which speeds up the shadowing procedure since the machine has a permanent vacuum. The handling and procedures are identical but it is recommended to demagnetize the tweezers to prevent the nickel grids from sticking to them.
8. The original powder stock of BAC was kindly provided by Bayer, Leverkusen, Germany. The chemical is now available also from different suppliers (i.e., Sigma, cat. B6295), but has never been tested for this specific application.
9. Tissue culture dishes are preferred over standard Petri dishes, as their surface treatment allows covering the surface with smaller volumes. A reduced hypophase volume improves the stability of the detergent-DNA film during the spreading (*see also* **Note 32**).
10. Bent tweezers (Fig. 3e and f): to hold the grids as flat as possible while touching the spreading surface, it is of great help to slightly bend the tweezers tips; this can be done mechanically applying pressure on the tips with a proper tool. Particular care is necessary to avoid the breakage of the tips. Recently, we also obtained satisfactory results using Dumont pinzettes (*see* Subheading 2.8).
11. Better results have been obtained with grid tables that fix individual grids by small pins (Fig. 4a), compared with those currently available on the market, where multiple grids are fixed on the table by metal stripes. Besides the ease in fixing/removing individual grids, the former tables show reduced interference with low angel rotary shadowing, by maximizing the area of each grid effectively exposed to the correct amount of evaporated Pt. Leica is currently considering to restart production of these grid tables. Magnetic grid holders can also be efficiently used, if Nichel grids are used (*see* **Note 7**).
12. The accurate determination of the very flat angle between the electron gun and specimen table is crucial to observe an optimal difference in thickness between ssDNA and dsDNA. To facilitate the determination and the reproducibility of the optimal angle in independent experiments, the knob available on the MED 020 to tilt the rocking rotary stage can be substituted with a precision rotation platform, providing micrometric control of this angle (Fig. 4a and d, black arrow).
13. A standard EM experiment, consisting of 100 replicating molecules (by a standard 1k × 1k resolution CCD camera), corresponds to several Gigabytes of digital files!

14. For experiments in *S. cerevisiae*, it is recommended to extract genomic DNA from cells synchronized in S-phase, i.e., 30–180 min after release from an α-factor arrest, depending on the treatment [5, 9]. While synchronization experiments have been performed also for EM analysis in mammalian cells—3-6 h after release from 16 h HU-block [32]—this does not seem to be strictly required. Although certainly more time consuming, it is possible to obtain 70–100 RIs from asynchronous, untreated U2OS cells or MEFs [15].
15. It is important to remove traces of growth media, which would otherwise absorb part of the monochromatic light in Subheadings 3.1, **step 4** and 3.2, **step 4**.
16. Due to the increasing Ethanol concentration in the suspension, cells tend to aggregate and deposit to the bottom of the dish. This does not interfere with the following extraction of genomic DNA, but requires extensive resuspension of the sample prior to each irradiation.
17. Timing of irradiation and number of crosslinking cycles strictly depend on the irradiance of the 365 nm bulbs. The observed irradiance within the crosslinker, measured by UV meter, is 6.2 mW/cm^2. This needs to be monitored, as it changes considerably with the type of apparatus, the age of the bulbs, and the distance of the samples from the bulbs. The total irradiation time should be adjusted to apply the same total irradiation (J/cm^2) to the samples as indicated here. Only doing so, it is possible to obtain optimal frequency of crosslinks on genomic DNA (1 crosslink in 80–90% of DNA linkers), particularly important for chromatin studies (*see* Subheading 3.13).
18. Incorporation of Cy3-dCTP can be monitored by visualization of nuclei using a standard fluorescence microscope. We consider an appropriate time for nuclear replication when 80–90% of the nuclei appear positive for Cy3 incorporation.
19. We have tested the efficiency of this RI enrichment protocol and compared it to standard BND cellulose-mediated enrichment [21], by performing a new round of digestion, RI enrichment, and EM analysis of several genomic DNA samples that had already been analyzed by EM [17], and for which sufficient amount of residual genomic DNA was still available in our stocks. For all the tested samples, we obtained practically identical results—in terms of ssDNA accumulation at forks and reversed fork frequency—to those previously obtained processing the same samples with the standard BND-cellulose enrichment (Fig. 6a in [17]). However, we reproducibly noticed that—despite similar density of total DNA on our EM grids—the identification of 70–100 RIs was significantly

(two- to threefold) faster than what typically observed with BND-enriched DNA. These observations strongly suggest that this optimized enrichment protocol allows "discarding" a higher fraction of linear, non-replicating DNA during the first elution step, yielding a higher enrichment of RI in the final elution, without introducing any bias on the type of replication structures and on ssDNA accumulation finally detected in the sample.

20. While choosing the restriction enzyme to digest genomic DNA, it is important to consider that: (a) frequent cutters will lead to smaller RIs (possibly complicating the analysis of phenomena happening at longer distance from the replication forks); (b) due to frequent methylation of CpG sequences, mammalian genomic DNA may be resistant to the action of a number of common restriction enzymes. It is also recommended to use enzymes releasing blunt DNA ends, to prevent ssDNA overhangs competition for binding to the BND cellulose.
21. It is important that residual RNA is removed prior to loading the sample on the QIAGEN-tip 20, to avoid interference with the enrichment procedure. This is particularly important for the extraction procedure described in Subheading 3.4, which normally leads to the extraction of large amounts of dsRNA, besides the expected genomic DNA. When residual RNA is detected on the gel, add to the restriction mix a few units of RNaseA (for ssRNA; 5 μl of 10 mg/ml in 250 μl reaction) and RNaseIII (for dsRNA; 1 μl of 1:100 dilution in TE in a 250 μl reaction; do not digest longer than 3 h to prevent DNA degradation). They are both usually active in the common restriction buffers.
22. The DNA recovered in Subheading 3.7, **step 12** is normally 1–15% of the total DNA loaded on the QIAGEN-tip 20 column, depending on the fraction of cells actively replicating their DNA. If the amount recovered at this step is far from this proportion, proper aliquots of the flow-through from Subheadings 3.7, **step 6** and 3.7, **step 7** can be loaded on a gel to figure out where the DNA got lost, recover it and repeat the procedure with appropriate modifications. The accurate quantification of the DNA recovered in Subheading 3.7, **step 12** requires an agarose gel, as the caffeine "contamination" in the RIs-enriched fraction interferes with standard spectrophotometric or fluorimetric measurements of DNA concentration.
23. The readout is calculated correcting for the different position of the quartz sensor in respect to the specimen (tooling factor). With the quartz crystal placed next to the sample (*see* Fig. 4b) the tooling factor in the QSG 100 should be set to 1. Different

settings will require the empirical determination of the correct tooling factor.

24. After two-to-three evaporation sessions, the electron gun overheats. Because of the high temperature, carbon films tend to strongly adhere to the mica sheet and are resistant to the floating procedure in Subheading 3.8, **step 7**.
25. Do not proceed lowering the mica in the water unless the carbon film is visibly detaching from the mica sheet. The proper coordination of mica sheet submersion and carbon film detachment is crucial for carbon film integrity and requires some manual skills and experience.
26. Generally, for each DNA spreading, grids covered with two-to-three different carbon films are used. As each carbon film has different absorption properties, this increases the chances of obtaining at least some grids with an optimal concentration of absorbed DNA molecules (*see* **Note 31**).
27. Formamide acts as a "partially-denaturing" agent, helping to disentangle and unfold DNA molecules during the spreading procedures. These conditions are optimal for psoralen-crosslinked DNA, which is inherently resistant to denaturation; if uncrosslinked DNA needs to be used, formamide concentration can be reduced to prevent DNA denaturation. If the DNA is only briefly exposed to formamide during the procedure (*see* **Note 30**), the vast majority of the psoralen-crosslinked DNA molecules is still visible as dsDNA, although some "breathing" is occasionally detected along the duplex and especially at DNA ends. Full denaturation of the sample requires longer exposure to these agents at higher temperatures (*see* Subheading 3.9.2).
28. The mix of the two droplets (by spinning briefly, *see* Subheading 3.9.1, **step 5**) should only be performed immediately before the spreading and having all necessary material ready, to minimize DNA exposure to formamide (*see* **Note 29**).
29. The final concentration of the DNA on the grid is crucial for a proper analysis of RIs. This depends on the DNA concentration in the sample, but also on the carbon absorption properties and the size of the spreading surface. Therefore, the spreading procedure often needs to be repeated to obtain grids with an optimal distribution of DNA molecules. For DNA samples that are particularly diluted, the DNA volume can be increased up to 5 μl, provided that formamide/glyoxal volumes are changed accordingly. BAC/plasmid volumes are unchanged, so that the size of the spreading surface and the density of marker molecules are not changed, leading to a higher frequency of DNA molecules of interest on the EM grids.

30. The stability of the monolayer can be heavily affected by any local perturbation of the conditions (hitting the bench, colleagues walking by, etc.). It is therefore highly recommended to perform the BAC spreading procedure in a quiet laboratory, on an (at least transiently) isolated bench. It is helpful to protect the working area with a Plexiglas box, open only to the side facing the operator.
31. The DNA molecules accumulate in close proximity to the edge of the spreading surface (close to the graphite powder).
32. Ethanol traces on the tweezers heavily disturb the spreading surface!
33. It is unavoidable that the monolayer surface will shrink and be perturbed while repeatedly touching it with the grids. The concentration of the DNA can also vary accordingly. Nonetheless, if the spreading surface is clearly unstable from the beginning, it is recommended to repeat the spreading procedure (*see also* **Notes 9** and **32**, on how to improve the spreading surface stability).
34. Denatured DNA is more difficult to absorb to the carbon films. It may happen that carbon-coated grids that proved proficient in absorbing dsDNA do not perform equally well absorbing denatured (mostly single-stranded) DNA (*see* **Note 37**). Due to the lower thickness, denatured DNA is also more difficult to visualize at the EM and usually requires optimal contrast both from uranyl acetate staining and from Pt/C rotary shadowing.
35. It is important that the plasmid marker is added only after denaturation. Preserving its duplex status, the marker will serve as an absorption control for the carbon films and will help assessing the quality of the contrast obtained on the grids (*see* Subheading 3.11, **step 2**).
36. Standard settings for platinum shadowing of a MED020 evaporation machine are 1.70 kV, 0.40 mA and tooling factor 1.0 and for carbon evaporation 1.70 kv, 0.70 mA and tooling factor 0.5. These values can differ from machine to machine but can be used as a starting point to determine your machine's settings.
37. A second round of platinum shadowing provides increase contrast to DNA molecules on carbon-coated grids. It is essential to wait that the Pt/C gun cools down, before the second round of platinum coating is performed.

Acknowledgments

We are grateful to José M. Sogo for his crucial support while learning all technicalities of this approach. We wish to thank the whole team at

the ZMB (Center for Microscopy and Image Analysis of the University Zurich) for consistently excellent technical assistance, while running our EM experiments. We are grateful to Arnab Ray Chaudhuri, Yoshitami Hashimoto, Fabio Puddu, and Vincenzo Costanzo for their assistance in optimizing this EM approach on Xenopus egg extracts. We also wish to thank Petr Cejka for suggesting the use of QIAGEN columns as possible valuable alternative to BND cellulose for the enrichment of a subpopulation of DNA molecules based on differential ssDNA content. We are also grateful to Sebastian Ursich for his recent efforts optimizing this approach and for careful reading of the manuscript. Work in the Lopes lab is currently financed by the SNF grant 31003A_169959, the ERC Consolidator grant 617102 (ReStreCa) and the Swiss Cancer League grant KFS-3967-08-2016.

References

1. Inciarte MR, Salas M, Sogo JM (1980) Structure of replicating DNA molecules of Bacillus subtilis bacteriophage phi 29. J Virol 34:187–199
2. Lucchini R, Sogo JM (1995) Replication of transcriptionally active chromatin. Nature 374:276–280
3. Sogo JM, Stahl H, Koller T, Knippers R (1986) Structure of replicating simian virus 40 minichromosomes. The replication fork, core histone segregation and terminal structures. J Mol Biol 189:189–204
4. Avemann K, Knippers R, Koller T, Sogo JM (1988) Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. Mol Cell Biol 8:3026–3034
5. Lopes M, Foiani M, Sogo JM (2006) Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. Mol Cell 21:15–27
6. Engels K, Giannattasio M, Muzi-Falconi M et al (2011) 14-3-3 proteins regulate exonuclease 1-dependent processing of stalled replication forks. PLoS Genet 7:e1001367. doi:10.1371/journal.pgen.1001367
7. Giannattasio M, Follonier C, Tourriere H et al (2010) Exo1 competes with repair synthesis, converts NER intermediates to long ssDNA gaps, and promotes checkpoint activation. Mol Cell 40:50–62. doi:10.1016/j.molcel.2010.09.004
8. Hashimoto Y, Chaudhuri AR, Lopes M, Costanzo V (2010) Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. Nat Struct Mol Biol 17:1305–1311. doi:10.1038/nsmb.1927
9. Sogo J, Lopes M, Foiani M (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science (New York, NY) 297:599–602
10. Berti M, Chaudhuri AR, Thangavel S et al (2013) Human RECQ1 promotes restart of replication forks reversed by DNA topoisomerase I inhibition. Nat Struct Mol Biol 20:347–354. doi:10.1038/nsmb.2501
11. Follonier C, Oehler J, Herrador R, Lopes M (2013) Friedreich's ataxia-associated GAA repeats induce replication-fork reversal and unusual molecular junctions. Nat Struct Mol Biol. doi:10.1038/nsmb.2520
12. Neelsen KJ, Zanini IMY, Mijic S et al (2013) Deregulated origin licensing leads to chromosomal breaks by rereplication of a gapped DNA template. Genes Dev 27:2537–2542. doi:10.1101/gad.226373.113
13. Neelsen KJ, Zanini IMY, Herrador R, Lopes M (2013) Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates. J Cell Biol 200:699–708. doi:10.1128/MCB.24.16.7140-7150.2004
14. Neelsen KJ, Lopes M (2015) Replication fork reversal in eukaryotes: from dead end to dynamic response. Nat Rev Mol Cell Biol 16:207–220. doi:10.1038/nrm3935
15. Ray Chaudhuri A, Hashimoto Y, Herrador R et al (2012) Topoisomerase I poisoning results in PARP-mediated replication fork reversal. Nat Struct Mol Biol 19:417–423. doi:10.1038/nsmb.2258
16. Ray Chaudhuri A, Ahuja AK, Herrador R, Lopes M (2015) Poly(ADP-ribosyl) glycohydrolase prevents the accumulation of unusual replication structures during unperturbed S

phase. Mol Cell Biol 35:856–865. doi:10.1128/MCB.01077-14
17. Zellweger R, Dalcher D, Mutreja K et al (2015) Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. J Cell Biol 208:563–579. doi:10.1083/jcb.201406099
18. Thangavel S, Berti M, Levikova M et al (2015) DNA2 drives processing and restart of reversed replication forks in human cells. J Cell Biol 208:545–562. doi:10.1083/jcb.201406100
19. Fugger K, Mistrik M, Neelsen KJ et al (2015) FBH1 catalyzes regression of stalled replication forks. Cell Rep. doi:10.1016/j.celrep.2015.02.028
20. Lopes M (2009) Electron microscopy methods for studying in vivo DNA replication intermediates. Methods Mol Biol 521:605–631
21. Neelsen KJ, Chaudhuri AR, Follonier C et al (2014) Visualization and interpretation of eukaryotic DNA replication intermediates in vivo by electron microscopy. Methods Mol Biol 1094:177–208. doi:10.1007/978-1-62703-706-8_15
22. Gasser R, Koller T, Sogo JM (1996) The stability of nucleosomes at the replication fork. J Mol Biol 258:224–239
23. Gruss C, Wu J, Koller T, Sogo JM (1993) Disruption of the nucleosomes at the replication fork. EMBO J 12:4533–4545
24. Mejlvang J, Feng Y, Alabert C et al (2014) New histone supply regulates replication fork speed and PCNA unloading. J Cell Biol 204:29–43. doi:10.1083/jcb.201305017
25. Wellinger RE, Lucchini R, Dammann R, Sogo JM (1999) In vivo mapping of nucleosomes using psoralen-DNA crosslinking and primer extension. Methods Mol Biol 119:161–173
26. Vollenweider HJ, Sogo JM, Koller T (1975) A routine method for protein-free spreading of double- and single-stranded nucleic acid molecules. Proc Natl Acad Sci U S A 72:83–87
27. Sogo JM, Thoma F (1989) Electron microscopy of chromatin. Methods Enzymol 170:142–165
28. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY
29. Trenz K, Errico A, Costanzo V (2008) Plx1 is required for chromosomal DNA replication under stressful conditions. EMBO J 27:876–885. doi:10.1038/emboj.2008.29
30. Ahuja AK, Jodkowska K, Teloni F et al (2016) A short G1 phase imposes constitutive replication stress and fork remodelling in mouse embryonic stem cells. Nat Commun 7:10660. doi:10.1038/ncomms10660
31. Sogo JM (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 297:599–602. doi:10.1126/science.1074023
32. Mojas N, Lopes M, Jiricny J (2007) Mismatch repair-dependent processing of methylation damage gives rise to persistent single-stranded gaps in newly replicated DNA. Genes Dev 21:3342–3355